13. (Once Amended) An isolated polypeptide comprising an alpha subunit of a cation channel, the polypeptide:

B1

(i) forming, with at least one additional HAC alpha subunit, a cation channel having the characteristic of activation upon hyperpolarization; and

(ii) having an amino acid sequence that has greater than about 96% identity to SEQ ID NO:1.

REMARKS

Claims 13 and 15-18 are pending in the present application and under examination. Appendix A shows changes made to the amended claims. Appendix B shows all pending claims currently under examination. For convenience, the Examiner's rejections are addressed in the order in which they were presented in the April 9, 2002 Office Action.

Status of the Claims

Claim 1 has been amended to recite sequence identity language over the full length SEQ ID NO:1. This amendment adds no new matter. Support for this amendment can be found, e.g., in the claims as filed and in the specification on page 4, lines 13-15.

Rejection under 35 U.S.C. § 101

Pending claims 13 and 15-18 stand rejected for lacking utility because the claimed invention allegedly lacks either a specific substantial asserted utility or a well-established utility. The Applicant respectfully traverses. The Applicant asserts that the present invention, the identification of polypeptides of the Hac3 channel, has utility. The identification of Hac3 channel polypeptides has utility because it makes possible the

routine identification of agonists and antagonists of the Hac3 channel, e.g., for the treatment of CNS diseases related to cell excitability.

With this amendment, Applicant attaches an expert declaration under 37 C.F.R. § 1.132 by Dr. Neil Castle, Associate Director of Biology at Icagen, Inc (*See* Exhibit A for declaration and Exhibit B for curriculum vitae of Dr. Castle). This declaration was originally submitted in the parent application, U.S. Application Number 09/548,933, in order to demonstrate the utility of nucleic acids encoding a Hac3 channel. Because polypeptides of a Hac3 channel have the same utility as nucleic acids encoding a Hac3 channel, this declaration is submitted herewith in order to demonstrate the utility of the polypeptides of the present invention.

A. Introduction

According to the MPEP, in order to assess utility, the Examiner should review the specification to determine if there are any statements asserting that the claimed invention is useful for any particular purpose. An invention has utility if the utility is specific, substantial and credible. A utility is specific if it is specific to the subject matter claimed. A utility is substantial if it has a real-world use. A utility is credible if it would be believable to one of skill in the art. In most cases, an applicant's assertion of utility creates a presumption of utility that is sufficient to satisfy the utility requirement of 35 U.S.C. § 101.

A *prima facie* showing of lack of utility by the Examiner must establish that it is more likely than not that a person of ordinary skill in the art would not consider that any utility asserted by the applicant would be specific and substantial. The present application claims Hac3 channel polypeptides. After reading the application, the skilled practitioner would appreciate that the Hac3 channel modulates cell excitability in the CNS. In addition, the skilled practitioner would (1) know how to routinely identify agonists or antagonists of Hac3 channels using the claimed amino acid sequences and the disclosed methods for activation of Hac3 channels, and (2) understand that antagonists or

agonists of Hac3 channels are useful for modulating cell excitability and in controlling CNS diseases related to cell excitability.

B. Examiner's Rejections

In the April 9, 2002 Office Action, the Examiner alleges that the instant specification fails to describe the practical utility of the claimed invention. The Examiner concedes that the claimed polypeptides are Hac3 channel polypeptides yet maintains that the polypeptides are of unknown function . According to the Examiner, there is no evidence showing that the claimed Hac3 polypeptides are associated with any physiological function or that they are associated with any known compounds, specific effects, or known disorders or diseases. The Examiner mistakenly understands Applicant's statement, "isolation of human Hac3 is therefore desirable, to better understand the physiology of Hac3 in humans and for the development of therapeutic and diagnostic applications to diseases related to hHac3 in humans" to suggest that Hac3 polypeptides are of unknown physiological function thereby making any utility associated with the claimed polypeptides possible only with further research.

The Applicant respectfully traverses. In a Declaration under 37 C.F.R. § 1.132, originally submitted in the parent application and submitted herewith, Dr. Neil Castle explains that Hac3 nucleic acids have a physiological role and function. According to Dr. Castle, Hac3 channels modulate cell excitability in the CNS by depolarizing resting potential in a cell and by directly causing excitatory rebound potentials in response to hyperpolarization (see attached declaration). Furthermore, Dr. Castle explains that the identification of Hac3 nucleic acids, coupled with the methods disclosed in the specification, and the level of skill in the art of ion channels, enable routine identification of agonists and antagonists of Hac3 channels. According to Dr. Castle, agonists and antagonists of Hac3 channels are useful for modulating cell excitability and in controlling CNS diseases related to cell excitability.

Dr. Castle's declaration focuses on Hac3 nucleic acids and not polypeptides because the parent application claimed the nucleic acids. The skilled

practitioner would understand that the same utility described for nucleic acids encoding a Hac3 channel applies to the polypeptides that they encode.

The Applicant, therefore, submits that the claimed polypeptides have a specific, substantial and credible utility.

C. The Hac3 channel is a hyperpolarization activated cation channel that modulates cell excitability.

In the present Office Action, the Examiner does not dispute Applicant's assertion that the claimed polypeptides are newly discovered Hac3 channel polypeptides. The Examiner disputes Applicant's assertion that the polypeptides have a practical utility. The Applicant respectfully traverses. The Applicant respectfully traverses because the Examiner provides no reasons why one of skill in the art, after reading the present specification, would not believe the Hac3 channel to be a hyperpolarized activated cation channel useful for modulating cell excitability. The Hac3 channel is a *hyperpolarized activated cation channel* that is widely expressed in the central nervous system. It is well known in the art that hyperpolarized activated channels expressed in the CNS modulate neuronal, and therefore, cell, excitability (*See* Pape, *Ann Rev Physiol,* 58:299-327, 1996, Exhibit C). The attached declaration of Dr. Castle demonstrates that the skilled practitioner, after reading the present specification, including Figures 3 and 4, would believe that the Hac3 hyperpolarized activated cation channel modulates neuronal, and therefore, cell excitability. As shown in figure 3, the Applicant expressed human Hac3 according to standard methodology in *Xenopus* oocytes and demonstrated that the claimed polypeptides comprise a hyperpolarized activated cation channel. Accordingly, the Applicant submits that the claimed polypeptides comprise a hyperpolarized activated cation channel useful for modulating cell excitability.

D. The Hac3 channel's physiological role is to modulate cell excitability by depolarizing resting potential in a cell and by directly causing excitatory rebound potentials in response to hyperpolarization.

The Hac3 channel's physiological role is to modulate cell excitability in the CNS by depolarizing resting potential in a cell and by directly causing excitatory rebound potentials in response to hyperpolarization (See Pape, *Id.*, and attached declaration). Because the Hac3 channel is capable of modulating cell excitability, the Hac3 channel is a useful target for the treatment of diseases and conditions caused by altered neuronal and cell excitability in the CNS. For example, blockers of Hac3 can be expected to decrease CNS activity (see attached declaration). Thus, blockers of Hac3 channels have utility for the treatment of diseases of hyperexcitability, such as epilepsy and migraine.

The present application provides amino acid sequences of Hac3 channels, routine methods of activating Hac3 channels, and routine methods of assaying for blockers of Hac3 channels. The skilled practitioner, after reading the present application, would therefore be able to (1) routinely identify antagonists of Hac3 channels and (2) determine if a putative antagonist of a Hac3 channel decreases CNS activity. Treating diseases related to cell excitability by targeting ion channels is a well known strategy in the art. For example, many currently marketed epilepsy modulate cell excitability by targeting excitatory ion channels with similarly broad distributions in the CNS. Antagonists of Hac3 channels, therefore, have utility for (1) decreasing CNS activity, (2) modulating cell excitability, and (3) treating diseases of hyperexcitability, such as epilepsy and migraines.

E. After reading the present application, the skilled practitioner would know how to identify Hac3 channel agonists and antagonists useful for modulating cell excitability.

It is well known in the art that once an ion channel has been identified, agonists or antagonists of the ion channels can be routinely identified. The present application provides Hac3 channel polypeptides. The present application also provides methods for activating a Hac3 channel. As provided in the specification, the Hac3 channel is activated by changes in voltage. Hac3 currents can be elicited by the application of voltage to cells expressing Hac3. Agonists and antagonists of Hac3 can routinely be identified by applying compounds to Hac3-expressing cells while applying voltage to the cells expressing Hac3 and measuring the effect on the magnitude of the Hac3 current. The blockage of the Hac3 current by cesium shown in figure 4 provides a direct example of the identification of an antagonist of the Hac3 channel. After reading the present application, the skilled practitioner, therefore, would know how to identify Hac3 channel agonists and antagonists useful for modulating cell excitability.

F. The identification of the Hac3 channel is useful (1) for modulating cell excitability (2) for identifying Hac3 channel agonists or antagonists capable of modulating Hac3 channels, and (3) as a target for treating diseases of the CNS.

There are many instances where modulation of an ion channel is useful for treating a specific disease even though the channel itself may not cause disease. For example, hypertension can be caused by a variety of illnesses such as renal disease and diabetes. Among common treatment strategies for hypertension is the use of drugs such as calcium channel blockers to relax the vasculature. Relaxing the vasculature to reduce blood pressure is useful and effective, even if the original cause of the hypertension is unrelated to vascular tone. Similarly, it is perfectly reasonable to expect that the targeting of Hac3, a hyperpolarization-activated cation channel widely expressed in the CNS, is an

appropriate strategy for suppressing hyperexcitability in diseases such as epilepsy without respect to the original cause of the condition.

G. The demonstration that Hac3 channels are functional hyperpolarization activated channels, coupled with the methods of modulating Hac3 channels provided in the specification and the level of skill in the art, is sufficient to demonstrate specific, substantial, and credible utility.

The Applicant maintains that the demonstration that the Hac3 channel is a functional hyperpolarization activated channel, coupled with the methods disclosed in the specification and the level of skill in the art, is sufficient to demonstrate specific, substantial and credible utility.

Specific utility

The Applicant asserts that the present invention has a specific utility. Specific utility is defined by the MPEP as a utility that is specific to the subject matter claimed. The MPEP explains that applications show sufficient specific utility when applicants disclose a "specific biological activity" and reasonably correlate that activity to a "disease condition." MPEP 2107.01, 2107.02. In this application, the Applicant discloses a "disease condition", cell hyperexcitability, that correlates with a "biological activity", the opening and closing of Hac3 channels. This application demonstrates that Hac3 channels modulate cell excitability. This application provides methods of identifying agonists and antagonists of Hac3 channels capable of modulating Hac3 channels e.g., for the treatment of CNS diseases characterized by neuronal and/or cell hyperexcitability, for example, epilepsy or migraines. The Applicant therefore submits that the present invention has a specific utility, e.g., identification of Hac3 channels that influence cell excitability in the CNS.

Substantial utility

The Applicant also asserts that the present invention has a substantial or "real world" use. This invention provides Hac3 channels polypeptides. The application also demonstrates that Hac3 channels modulate cell excitability. This application therefore has real world use in the modulation of cell excitability and in the identification of compounds that modulate the Hac3 channel. As previously discussed, it is well known in the art that once an ion channel has been identified, agonists or antagonists of the ion channels can be routinely identified. Throughout the specification, the Applicant teaches how to activate the Hac3 channel and how to identify agonists and antagonists of the Hac3 channel. For example, on page 44 of the specification to page 46, the Applicant provides assays that can be used to test for inhibitors and activators of the Hac3 channel, e.g., assays that involve measuring current, measuring membrane potential, measuring ion flux, or measuring patch-clamp electrophysiology. The Applicant therefore submits that the present invention has a substantial utility, e.g., the identification of Hac3 channels that modulate cell excitability, thereby making routine the identification of agonists or antagonists of Hac3 channels useful for treating diseases of hyperexcitability.

Credible utility

Finally, The Applicant asserts that the present invention has a credible utility. According to the MPEP, when an applicant has specifically asserted that an invention has a particular utility, that assertion cannot simply be dismissed by Office Personnel as being "wrong," even when there is reason to believe that the assertion is not entirely accurate. Rather Office Personnel must determine if the assertion of utility is credible (i.e., whether the assertion of utility is believable to a person of ordinary skill in the art based on the totality of the evidence and reasoning provided) MPEP 2107.02 III B. The Applicant submits that one of skill in the art, after reading this application, would (a) know how to identify Hac3 channels (b) know how to identify agonists or antagonists of Hac3 channels (c) know how to use those agonists or antagonists to modulate cell excitability. Because many currently marketed drugs treat CNS diseases of hyperexcitability, e.g., epilepsy, by targeting ion channels, the skilled practitioner would

believe that the identification of a new hyperpolarized activated cation channel is useful.

Accordingly, the Applicant respectfully requests that the utility rejection under 35 U.S.C. § 101 be withdrawn.

Rejection under 35 U.S.C. § 112, first paragraph

Pending claims 13 and 15-18 were rejected as not being enabled as the invention is allegedly not supported by either a clear asserted utility or a well established utility. The Applicant respectfully traverses the rejection. As described above, the invention claimed in the present application is supported by a specific, substantial, and credible utility. The specification provides methods of identifying the claimed polypeptides, methods of activating and blocking Hac3 channels, and methods of identifying agonists and antagonists of Hac3 channels. Accordingly, the Applicant requests that the rejection under 35 U.S.C. § 112 be withdrawn.

Rejection under 35 U.S.C. § 112, written description

Pending claims 13 and 15-18 were rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention. The Examiner has rejected the claims for two reasons. First, because the specification allegedly fails to describe the entire genus of proteins encompassed by the claims and second, because the specification allegedly fails to provide a complete structure of the claimed polypeptides or other relevant identifying characteristics sufficient to describe the claimed invention in clear and concise terms.

To the extent that the rejection applies to the claims as amended, the Applicant respectfully traverses. The claims fully comply with the requirements for written description of a chemical genus as set forth in *University of California v. Eli Lilly & Co.*, 43 USPQ2d 1398 (Fed. Cir. 1997). As described by the Federal Circuit in *Lilly*,

"[a] description of a genus of cDNAs may be achieved by means of . . . a recitation of structural features common to the members of the genus . . . ." *Lilly*, 43 USPQ2d at 1406. Furthermore, the court in *Fiers v. Revel* stated that an adequate written description "requires a precise definition, such as by structure, formula, chemical name, or physical properties." *Fiers v. Revel*, 25 USPQ2d 1601, 1606 (Fed. Cir. 1993).

The present invention relates to the discovery of polypeptides of a novel cation channel, Hac3. The polypeptides are claimed by reference to shared structural features, *i.e.*, percent identity to the sequence disclosed in SEQ ID NO:1. The polypeptides are also claimed by reference to shared functional features, *i.e.*, the ability to form a cation channel having the characteristic of activation upon hyperpolarization.

The percent identity of a polypeptide to a reference sequence is a structural property of the polypeptide because it relies entirely upon the amino acid sequence of the molecule (*see, e.g.*, Sambrook, *Molecular Cloning: A Laboratory Manual*, pp. 9.47-9.51 (2nd ed. 1989); *see also* Stryer, *Biochemistry*, pp. 573 (2nd ed. 1975)). Furthermore, the recitation of sequence identity in the claims makes the identification of the claimed polypeptides easily accomplished by one of skill in the art. Algorithms for determining percent sequence identity and sequence similarity for the identification of polypeptides are well known to those of skill in molecular biology and are described in the specification on pages 22 to 24.

Furthermore, as well as providing a reference sequence identification number for the polypeptides of the present invention, the specification also provides functional assays for the identification of the polypeptides of the present invention. On page 11, line 27 to page 12, line 8, the specification teaches expressing or co-expressing the putative hHAC3 polypeptide monomer and examining whether the monomer forms a cation channel with Hac3 functional characteristics, such as activation upon hyperpolarization. This assay is used to confirm that a putative Hac3 polypeptide is actually a Hac3 polypeptide by examining the polypeptide's functional characteristics. A polypeptide with similar amino acid identity to Hac3 that forms a cation channel with Hac3 activity when expressed, shares the same functional characteristics of Hac3 and is

therefore, a species of Hac3. Conversely, a sequence that diverges even by only one amino acid is not a species of Hac3 if it doesn't form a cation channel when expressed. This assay allows one of skill in the art to identify the claimed polypeptides without undue experimentation.

In addition, in order to demonstrate Hac3's ability to form cation channels with activation upon hyperpolarization, the Applicant expressed Hac3 in *Xenopus* oocytes (*See* figures 3 and 4 of the specification). Figures 3 and 4 of the specification graph the activity of the Hac3 polypeptide.

Accordingly, in the present application, the Applicant has provided both reference amino acid sequences, as well functional characteristics. As required by the standard set forth in *University of California v. Eli Lilly*, these features are common to all of the members of the claimed Hac3 polypeptides. The conserved sequences encoding structural features of the genus and the functional requirements of the claimed polypeptides clearly allow persons of ordinary skill to recognize the polypeptides of the present invention. The specification thus appropriately describes the claimed Hac3 polypeptides using structural/physical features, as required by the court in *University of California v. Eli Lilly.* Because the specification clearly sets forth both functional and structural features common to the Hac3 polypeptides of the present invention, the Applicant requests that the rejection under 35 U.S.C. § 112, written description, be withdrawn.

CONCLUSION

In view of the foregoing, the Applicant believes all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,

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APPENDIX A

VERSION WITH MARKINGS TO SHOW CHANGES MADE

13. (Once Amended) An isolated polypeptide comprising an alpha subunit of a cation channel, the polypeptide:

(i) forming, with at least one additional HAC alpha subunit, a cation channel having the characteristic of activation upon hyperpolarization; and

(ii) having an amino acid sequence that has greater than about [75%] 96% identity to [amino acids 1-50 of] SEQ ID NO:1 [or greater than 90% identity to amino acids 640-775 of SEQ ID NO:1].

APPENDIX B
PENDING CLAIMS SUBJECT TO EXAMINATION

13. An isolated polypeptide comprising an alpha subunit of a cation channel, the polypeptide:

(i) forming, with at least one additional HAC alpha subunit, a cation channel having the characteristic of activation upon hyperpolarization; and

(ii) having an amino acid sequence that has greater than about 96% identity to SEQ ID NO:1.

15. The isolated polypeptide of claim 13, wherein the polypeptide has an amino acid sequence of SEQ ID NO:1.

16. The isolated polypeptide of claim 13, wherein the polypeptide comprises an alpha subunit of a homomeric cation channel.

17. The isolated polypeptide of claim 13, wherein the polypeptide comprises an alpha subunit of a heteromeric cation channel.

18. The isolated polypeptide of claim 13, wherein the polypeptide has a molecular weight between about 85 kDa to about 94 kDa.

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QUEER CURRENT AND PACEMAKER: The Hyperpolarization-Activated Cation Current in Neurons

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KEY WORDS: anomalous rectification, inward rectification, ionic currents, rhythmogenesis, cyclic AMP, nervous system

ABSTRACT

The cation conductance activated upon hyperpolarization of the membrane beyond the resting value appears to represent an ubiquitous type of membrane channel. Our understanding of the respective membrane current, termed I_h, in neurons has matured from that of a "queer" current toward that of a highly regulated mechanism that is particularly important in determining integrative behavior near rest and providing the pacemaker depolarization during rhythmic-oscillatory activity.

INTRODUCTION

The rich variety and complexity of functions of the nervous system are largely attained by a fine-tuned interplay between the intrinsic neuronal properties and the quality of synaptic interconnections. Intrinsic electrophysiological characteristics, in turn, reflect the type, location, and density of voltage- and ligand-gated ion channels that regulate the flow of ionic currents across the neuronal plasma membrane and that are controlled by a large variety of transmitter substances and intracellular messenger systems (67). In view of the highly specific—albeit dynamic—electroresponsiveness required of the neuronal elements, it is not unexpected that neurons possess a rich repertoire of ion channels, including classical types such as those producing voltage-dependent sodium (Na^+) and potassium (K^+) currents during the generation of an action potential (70), as well as a number of less conventional ionic conductances (85). A rather peculiar intrinsic mechanism, originally observed by Ito and

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co-workers (4, 72) in cat motoneurons, is evident as a slow relaxation of the potential change induced by hyperpolarizing current, producing non-ohmic behavior of the current/voltage (I/V) relation in the hyperpolarizing direction. The underlying, time-dependent membrane conductance was first identified in rod photoreceptors as a caesium (Cs^+)-sensitive, slow inward current that is turned on by hyperpolarization and is capable of depolarizing the membrane, which results in the typical sequence of an initial transient hyperpolarization followed by a slowly decaying component in response to bright flashes of light (5, 7, 8, 52). The current in photoreceptors was termed I_h (or I_A; 5) because of its activation by hyperpolarization. At about the same time, a similar current system was discovered in sino-atrial node cells and in Purkinje fibers of the mammalian heart (16, 17, 30, 31, 139), and it became apparent that the slow inward current represents a cation conductance permeable to Na^+ and K^+ ions. To underline its anomalous behavior, i.e. being an inward current activated by hyperpolarization that looked funnily similar to the presumed K^+ conductance I_{K2}, the current was named "funny" current (I_f). This current has since gained significant interest because of its involvement in the generation and control of spontaneous cardiac activity (see 20, 34, 36). Evidence for the existence of an equivalent current in central neurons was first reported by Halliwell & Adams (64). They observed in hippocampal pyramidal cells a slow inward current activation upon membrane hyperpolarization, which was termed "queer" current (I_q) in view of its odd electrophysiological behavior and its undefined functional significance. A current with similar properties was later found in a wide variety of neuronal and non-neuronal cells, and this hyperpolarization-activated current was recognized as an ubiquitous phenomenon within cells of the nervous system. It was called I_q, I_f, I_{AR} (anomalous rectifier), or I_{IR} (inward rectifier). However, to differentiate this current from the classical K^+ inward rectifier, the term hyperpolarization-activated current (I_h) is now preferred for use in the nervous system (67).

Although the respective neuronal channels were initially thought to be resistant to modulatory influences, accumulating data indicate that they are important targets for neurotransmitter and second messenger systems, which implies important physiological roles in the control of cellular electrical activities. The aim of this review is to summarize the experimental data on the most relevant biophysical properties of the hyperpolarization-activated channels and to discuss the mechanisms of regulatory influences that may contribute to the functional significance of I_h within the nervous system.

BIOPHYSICAL PROPERTIES OF I_h

The I_h current is typically seen as a slowly developing inward current activation upon hyperpolarization of the membrane beyond the resting potential. The rate

of activation and the amplitude of I_h increase with increasing hyperpolarization, which is apparent as a region of anomalous inward rectification when the current magnitude is plotted against membrane potential in an I/V relation. In an initial attempt to characterize the slow inward current relaxation, it was important to demonstrate that it behaved in a way expected of a time- and voltage-dependent membrane conductance, as opposed to arising from a shift in driving force due to ion accumulation or depletion. The strongest evidence in favor of a conventional ionic current comes from the work of DiFrancesco (35), who identified single-channel events of low conductance (approximately 1 pS) that underlie I_f in rabbit sino-atrial node cells. Although single I_h channels remain to be identified in neurons, studies of the macroscopic currents strongly suggest mediation by a voltage-gated membrane conductance (cf 134) in showing that (*a*) activation and deactivation are associated with increases and decreases in membrane conductance, respectively (e.g. 1, 91, 94, 122); (*b*) the envelope of tail currents closely matches the kinetics of current activation (e.g. 5, 66, 76, 91, 94), as is predicted by Hodgkin-Huxley (70) formalism for a voltage-dependent conductance; (*c*) the voltage dependence of steady-state activation is well described by a Boltzmann function in most preparations (e.g. 6, 10, 46, 66, 76, 83, 87, 91, 94, 115, 118, 128); and (*d*) the reversal potential does not change even in response to very large and long periods of current activation (e.g. 1, 6, 94, 125). Second, it is clear that anomalous rectification due to I_h is different from classical inward rectification, originally described by Katz (78) as a higher conductance for inward than for outward current in frog skeletal muscle fibers, and which has since been found in a large number of biological membranes (107). Different from I_h, the classical inward rectifier (*a*) is a pure K^+ conductance with no significant contribution of Na^+; (*b*) is sensitive to external barium ions (Ba^{2+}), in addition to Cs^+; (*c*) is active mainly negative to the K^+ equilibrium potential, with the activation range depending on the extracellular K^+ concentration; (*d*) activates within a few milliseconds of an appropriate change in membrane potential; and (*e*) is ohmic in nature with its voltage dependence arising secondary to voltage-dependent block by internal magnesium ions (Mg^{2+}) or polyamines (53, 58). Furthermore, inwardly rectifying K^+ channels have been cloned (e.g. 82), whereas the gene that encodes the I_h channel has not been identified. Both types of inward rectification, the K^+ conductance and the mixed Na^+/K^+ conductance underlying I_h, can exist in parallel in biological membranes (e.g. 124, 135, 137).

Gating of the I_h Channels

One peculiar property that distinguishes the hyperpolarization-activated inward current from many other voltage-activated conductances pathways is the slow, complex time course of activation and deactivation (for a review on I_f in cardiac

tissue, see 34, 36). Following hyperpolarizing voltage steps negative to approximately −50 mV, there is first an instantaneous current that is resistant to extracellular Cs^+ and possesses linear I/V relationships, which probably reflects the passage of current through multiple "leakage" conductance pathways. The I_h inward current turns on with a delay and slowly relaxes to a steady-state value, resulting in a characteristic sigmoidal shape of the current waveform (3, 7, 11, 30, 33, 46, 66, 91, 116). The delay is voltage dependent, decreasing with more negative membrane potentials, and reflects a process intrinsic to I_h activation (33, 116). The slow inward current following the delay is a function of voltage and time, with the rate of activation increasing with more negative values of the membrane potential. Current kinetics are satisfactorily described

slow single-exponential processes in most neuronal cell types in mammals ...5, 51, 76, 94, 96a, 117, 128, 137) and non-mammals (3, 24, 79, 125). Time constants are generally strongly voltage dependent (but see 118, 120), averaging (at 35°C) around 1 to 2 s at potentials near activation threshold and around 200 to 400 ms at the potential of maximal activation of I_h, if a Q_{10} of 3 to 4 (3, 48, 64, 125) is assumed. Two kinetically distinct components of I_h with time constants in the range of hundreds of milliseconds and seconds, respectively, whose relative amplitudes are not correlated, may indicate the existence of two distinct populations of I_h channels (10, 18, 116). This view is supported by the finding that the two kinetically different current components can be differentially expressed in one type of neuron (114). The relatively fast time course of activation of I_h in some classes of neurons (64, 87, 96a) may then reflect a predominance of the fast-current component. Two examples at the extreme end indicate the wide range of I_h kinetics: Descriptions assuming double exponential functions with time constants between tens and hundreds of milliseconds (at room temperature) have been found to be sufficient for I_h in rod (5, 66) and cone (88) photoreceptors, whereas the kinetics of I_h activation ...nge within the order of tens of seconds in slowly adapting lobster stretch receptor neurons (46).

Termination of the hyperpolarizing voltage steps evoke outward relaxations of the membrane current, indicating deactivation of the conductance underlying I_h. Envelopes of these tail currents, measured following hyperpolarizing steps of various durations, closely match the time course of the onset of I_h (5, 33, 66, 76, 91, 94, 134), as is expected for a voltage-dependent conductance (70). The deactivation of I_h exhibits a voltage dependence roughly opposite to that of activation kinetics, in that deactivation becomes faster as the membrane potential is depolarized, yielding a roughly bell-shaped voltage dependence of the rates of I_h on- and off-relaxations, with maximal time constants near the potential of 50% steady-state activation (11, 12, 46, 47, 66, 76, 91, 94, 116, 125). Kinetics of tail currents are mostly described by single-exponential functions, with time constants ranging in the order of hundreds of milliseconds,

although the exact kinetics were difficult to determine largely because of contamination by other voltage-dependent currents (12, 76, 91, 94, 116, 125). Detailed analyses of the kinetics in cardiac cells (33, 89, 134) and photoreceptors (46, 66) demonstrated that both activation and deactivation processes are sigmoidal functions of time at high negative and positive voltages, whereas exponential processes with one or two time constants satisfactorily describe the current kinetics in the vicinity of the half-activation voltage.

Decrements of the current during maintained hyperpolarization or alterations in the current waveform following activation after long depolarizing steps were not observed (6, 46, 94, 116, 125, 134), indicating a lack of inactivation of the underlying channels from open or closed states, respectively. Because I_h does not inactivate, measurement of the voltage dependence of steady-state activation is straightforward in that tail currents evoked upon return to various membrane potentials following maximal activation of I_h can be used. The steady-state activation of I_h is well described by a Boltzmann function in most preparations. The I_h currents begin to activate between −45 and −60 mV, and generally, half-activation is seen between −75 and −85 mV (3, 6, 10, 12, 18, 27, 46, 71, 83, 88, 91, 94, 96a, 115, 117, 118, 137), although closer to −65 mV in rod photoreceptors (66) and closer to −95 mV in some types of central and peripheral neurons (76, 87, 122, 125, 128). The I_h conductance is generally maximal at membrane potentials negative to −110 mV (but see 3). Maximal amplitudes vary between different types of neurons, with reported conductance values ranging in the order 2–10 nS (115, 125, 128). From available data with regard to total current production and surface area (19, 32), it can be inferred that at voltage levels of maximal activation, the density of I_h is between 1 and 5 $\mu A/cm^2$ (46). In view of a single-channel conductance at around 1 pS (35), the average channel density would be below 0.5 μm^{-2}, which is to be compared with an average Na^+ channel density of about 500 μm^{-2} (46). The steepness of the activation curve, however, will ensure that even small changes in membrane polarization result in activation of a substantial part of the channels, which can be assumed to largely account for the anomalous inward rectification observed in the whole-cell I/V-relation. The activated conductance itself is not rectifying, as indicated by the nearly linear I/V-relation of the fully activated current (3, 6, 38, 46, 87, 101, 115). Comparative interpretation and estimates of channel densities in different types of cells are complicated, however, largely because of the relative uncertainty about the cells' geometry and membrane surface area. Additional constraints in the interpretation of these quantitative measurements derive from the reported influence of external ions, transmitters, and second messenger systems on the I_h conductance or activation curve (as is described in detail below). These biological variables may help to explain the high variability of I_h observed in neurons of the same type (e.g. 51, 83) and the reported wide range of I_h activation in various preparations.

Another phenomenon to be considered in evaluating the voltage dependence of I_h is current rundown, attributable to the loss of intracellular elements that make the channels available to voltage-dependent activation and that are associated with a voltage range of activation of I_h different from that of a physiologically more intact cell (142).

The quantitative descriptions of I_h facilitated a variety of reaction schemes to account for the rather complex current kinetics. A modified Hodgkin-Huxley (70) scheme of second order largely described the sigmoidicity of current onset and deactivation in sino-atrial node cells (134), although a more complex model involving voltage-dependent transitions between five open states and three closed states more fully accounted for the current waveforms in cardiac Purkinje fibers (33). Modeling of I_h activation in corticotectal neurons of the [illegible], using a product of Hodgkin-Huxley type multistate reaction sequences, indicated the involvement of non-identical gating subunits and/or non-independence (cooperativity) in the gating reaction, with a minimum of two energetically non-equivalent conformational changes required for channel opening (116). Empirical quantifications of I_h activation kinetics in photoreceptors included Hodgkin-Huxley formalism involving three states with voltage-dependent and voltage-independent transitions (46) or transitions between two closed and three open states governed by voltage-dependent rate constants (11). Other theories found the simple summation of two independent exponential components of opposite polarity (66) or a kinetic scheme based on the cooperation of fast and slow current components (28) sufficient to account for the kinetics of I_h. The conclusion drawn from these models is that opening of the I_h channel requires prior transitions through multiple closed states and that the channel passes through several open states before closing; inactivated states are not significant. Furthermore, protein subunits may cooperatively interact within the gating processes.

Ionic Nature of I_h

The I_h current is a mixed cation current. The I_h channels are not, however, nonselective. For example, although the channel is permeable to Na^+, it is almost impermeable to the very similar ion lithium (Li^+) (permeability ratio $P_{Li}/P_{Na} = 0.06$; 136), which is unique in that no other type of channel can discriminate Li^+ from Na^+ (68). The permeability of I_h channels to K^+ is high compared with that of the similar ion rubidium (Rb^+) ($P_{Rb}/P_K = 0.55$; 136). Choline, protonated Tris, or larger amines such as tetramethylammonium or tetraethylammonium cannot pass through the I_h channel, and there is little permeability to ammonium ions, but I_h channels are permeable to thallium ions (Tl^+) ($P_{Tl}/P_K > 1.55$; 47, 136). Based upon permeabilities to organic cations, the apparent pore diameter of I_h channels has been estimated

to be between 4 and 4.6 Å (136). Thus it appears that the I_h channel is highly selective for Na^+ and K^+, and the I_h current will be carried by both Na^+ and K^+ under normal conditions. Reversal potentials of I_h are between approximately −50 and −20 mV and thus in a region positive to the normal resting potential, as has been estimated from analyses of tail currents or fully activated I/V-relations in different types of cells under physiologic conditions (6, 10, 12, 18, 25, 27, 30, 46, 66, 76, 79, 91, 94, 96a, 101, 115, 118, 122, 125, 128, 134). When the ratio of P_{Na}/P_K was explicitly calculated, values from 0.2 to 0.4 were found (46, 66, 88, 115) for moderate changes of Na^+ and K^+ concentrations from physiological levels, although the ratio can increase with more extreme changes in the extracellular levels of Na^+ and K^+ (46, 47). A prominent aspect of I_h characteristics implying a deviation of the conductance mechanism from the independence principle is an increase in current amplitude upon increases in the external K^+ concentration that is independent of the effects on the I_h reversal potential and that results in an increased slope of the I/V relation (6, 25, 27, 32, 47, 66, 76, 87–89, 94, 115, 118, 122, 124). The kinetic parameters and the steady-state voltage dependence of I_h are generally not affected (but see 88), suggesting a regulation of the conductance mechanism underlying I_h with no change in the gating properties (32, 47). A model of this phenomenon in corticotectal neurons predicted an approximately ±20% variation in I_h conductance during only a 1 mM change in extracellular K^+ concentration, and an ability of K^+ to maximally increase the I_h conductance approximately tenfold over basal levels (115). In contrast, current modifications upon changes in extracellular Na^+ levels are merely the result of the altered driving force, or may reflect changes in the K^+ gradient secondary to a severe reduction in the external Na^+ concentration (118). A quantitative analysis performed in rod photoreceptors (66) demonstrated that the maximal I_h conductance depends on the square root of the external K^+ concentration, with the flux of both Na^+ and K^+ ions being affected (see also 47). Following from this is the peculiar situation that Na^+ is necessary as the carrier of inward current in I_h channels at a range of membrane potential where I_h is important physiologically, yet K^+ is required for the channels to carry any current (32, 56, 56a, 136). Our understanding of the underlying permeation pathway is incomplete. The I_h channel is considered a multi-ion pore possessing a high-affinity external binding site, the affinity of which is modulated by K^+ (32, 66, 68). Based on the efficacy of block by different ions and the degree to which Na^+ and K^+ antagonize the block, the permeation pathway of the I_h channel was seen to contain at least two binding sites: one having a higher affinity for K^+ and another having a higher affinity for Na^+ (135a).

Experiments aimed at examining the permeability of chloride ions (Cl^-) through the I_h channels were initially puzzling. Shifts in the Cl^- equilibrium

potential through replacement of extracellular choline chloride with sucrose or mannitol, or injection of Cl^- into the cells, did not significantly affect I_h (25, 115, 118), whereas substitution of external Cl^- by larger anions such as isethionate resulted in a substantial reduction in current amplitude and no effects on the I/V relation (76, 91, 94, 122, 139). Because the current amplitude decreased, despite an increase in driving force on Cl^-, the large anion substitutes were thought to exert an unspecific blocking action on the I_h conductance. A study of the equivalent conductance in isolated sino-atrial node cells (56) corroborated the reduction in current amplitude by large anions and in addition demonstrated that replacement of Cl^- by small anions such as iodide or nitrate supported an intact current. The underlying conductance was found to be a saturating function of the extracellular Cl^- concentration, indicating an activating effect of small anions rather than a blocking effect of large anions (56). Therefore, it appears that the I_h conductance is unique because it is carried by Na^+ and K^+, yet it is dependent on an extracellular anion (Cl^-). Cl^- may perform a screening role for cations bound at external sites of the multi-ion channel and thereby represent a necessary step in channel permeation by cations (56). Although the response of I_h to changes in the ionic environment supports the notion that the channels' gating action and ion conduction are associated with operationally independent channel structures (48), the proposed mechanisms and models await further experimental clarification (e.g by single-channel analysis) and thus far can only be regarded as a useful description of available results (32).

Blockade of the I_h Channels

As originally described in photoreceptor cells (52), a general feature of the I_h current is its sensitivity to low concentrations (0.1–5 mM) of extracellular Cs^+. In fact, the blockade by extracellular Cs^+ and the relative insensitivity to Ba^{2+} has largely facilitated the isolation of the hyperpolarization-activated cation current and the evaluation of its physiological roles in a large variety of preparations. Internal Cs^+ does not block (11, 91, 96a, 118), whereas external Cs^+ results in a concentration- and voltage-dependent blockade, with dissociation contants at zero membrane potential ranging between 0.5 and 3.7 mM in cardiac cells (32). Cs^+ presumably binds to a blocking site inside the channel that is different from the externally located regulatory site to which K^+ binds (32). Cs^+ blocks current in negative regions of the I/V-curve, but has no effect or even enhances current positive to the reversal potential (32). These findings, and the known effect of Cs^+ to block other types of voltage-dependent conductances, such as delayed rectifier and K^+ inward rectifier channels (67, 107), put some constraints on the exclusive use of Cs^+ when the contribution of the I_h current to electrical neuronal activity is to be quantitatively analyzed. In addition, manganese (Mn^{2+}) and Ba^{2+}, at millimolar concentrations often used to block contaminating Ca^{2+} and K^+ currents during the study of I_h, induce a positive and negative shift in the activation curve of the respective current in sino-atrial node myocytes (40). This result may help to explain reported reductions in I_h amplitude following application of Ba^{2+} (12, 14, 76, 122). Strontium also blocks I_f in cardiac cells, whereas Ca^{2+} is a very weak blocker (but may modulate I_h activation), and Mg^{2+} has little effect on the hyperpolarization-activated current (99). The neuronal I_h has proven to be largely unaffected by extracellular tetrodotoxin, cobalt (Co^{2+}), tetraethylammonium, and 4-aminopyridine in concentrations that effectively blocked Na^+ and K^+ conductances (25, 27, 46, 88, 96a, 115, 120, 122, 125, 128), although intracellular tetraethylammonium at <15 mM blocked I_h in cultured neocortical neurons (18). Volatile anesthetics, such as enflurane and halothane, evoke a negative shift in the I_h activation curve and reduce the maximal I_h conductance in bullfrog sensory neurons (126), and intracellular QX-314 (5-10 mM), a quaternary derivative of the local anesthetic lidocaine, blocks I_h completely throughout its activation range in hippocampal CA1 pyramidal cells (103a). Rb^+ induces a voltage-independent block of the I_h conductance in photoreceptor and cardiac cells (32, 47), although Rb^+ is far less potent than Cs^+ (32), and Rb^+ can also permeate the I_h channel (136). An agent, 9-amino-1,2,3,4-tetrahydroacridine, was found to block the hyperpolarization-activated current in hippocampal pyramidal neurons (65) and cardiac cells (41) in a voltage-independent manner and at lower concentrations than other known I_h channel blockers (IC_{50} at 20 and 300 μM in the two preparations, respectively), although it similarly reduced agonist-induced or delayed rectifier K^+ conductances. A group of substances termed specific bradycardiac agents in view of their negative chronotropic effects exert a potent inhibition of the hyperpolarization-activated cation current in cardiac cells (37, 131–133) and mammalian central neurons (101). Depending on the type of drug, inhibition results from a reduction in I_h conductance and a negative shift of I_h activation or a use-dependent block of the open channels, with no alteration in the gating properties. Although these drugs potently inhibit I_h, their selectivity under various experimental conditions is still a matter of controversy (101, 131). Finally, a depressant effect on the gating process of I_h with some physiological significance was obtained with H^+ ions in lobster stretch receptor neurons (48), which may underlie reported alterations in anomalous inward rectification upon manipulations of the buffered extracellular medium (22).

The complete pharmacological profile of the neuronal I_h channels remains to be developed in detail, and a more specific and efficient I_h blocker would be desirable for a quantitative analysis of the physiological roles of the I_h conductance in the different classes of cells.

REGULATION OF I_h

An important and physiologically significant property of I_h channels is their ability to be regulated by neurotransmitters and metabolic stimuli. Although the control of cardiac I_f channels through the intracellular cyclic adenosine monophosphate (cAMP) system has been known for several years (34, 36), early studies on neuronal I_h found no changes attributable to neurotransmitters (e.g. 11, 64). However, the conclusions relied on the test of a relatively small number of cells and compounds. In fact, later experiments in thalamo-cortical neurons (103) and neurons of the brain stem nucleus prepositus hypoglossi (15) presented evidence of an increase in I_h activation upon stimulation of β-adrenergic and serotonergic receptors. The modulation relies on a positive shift of the voltage dependence of the I_h activation curve by up to +10 mV, with no changes in the steepness of the curve or in the fully activated I/V-relation. The intracellular messenger system mediating this response most likely involves stimulation of adenylyl cyclase and a resulting increase in intracellular cAMP level, as was indicated by the mimicking effects exerted by membrane-permeable analogues of cAMP (8-bromo-cAMP; dibutyryl-cAMP), a stimulant of adenylyl cyclase activity (forskolin; Figure 1*A*), or a phosphodiesterase inhibitor (3-isobutyl-1-methyl-xanthine) (15, 51, 95, 103, 125). Subsequent studies suggested that this intracellular pathway and associated modulation of I_h channels is shared by a subset of receptor types that are positively coupled to adenylyl cyclase activity in various types of cells (Figure 1*B*), including serotonergic receptors of an as yet unclassified subtype in mammalian and crustacean motoneurons (58, 79, 83, 123), substantia nigra pars compacta neurons (97) and salivary gland cells of the leech (138), noradrenergic receptors of the β-subtype in neurons of the medial nucleus of the trapezoid body (10), and histaminergic H_2 receptors in thalamic neurons (96).

Furthermore, nitric oxide (NO), a gaseous messenger molecule known to induce a rise in the intracellular level of cyclic guanosine monophosphate (cGMP) through direct stimulation of guanylyl cyclase activity (26), induced a positive shift in I_h activation similar to that observed after stimulation of the more classical receptors in thalamic neurons (102) and endothelial cells of the blood-brain barrier (73). These findings raise the interesting possibility that the cAMP and the cGMP systems are synergistically controlling the I_h conductance (Figure 1*B*; 102), for example, via a direct modulation of the channels (42) or a cGMP-regulated isozyme of cyclic nucleotide phosphodiesterase (13). In support of this hypothesis is the finding that the NO effect on I_h is imitated by membrane permeable analogues of both cAMP and cGMP (73, 102) and prevented by an inhibitor of adenylyl cyclase activity (102).

Receptors that are negatively coupled to adenylyl cyclase, in turn, are capable of negatively shifting I_h activation by up to −10 mV along the voltage

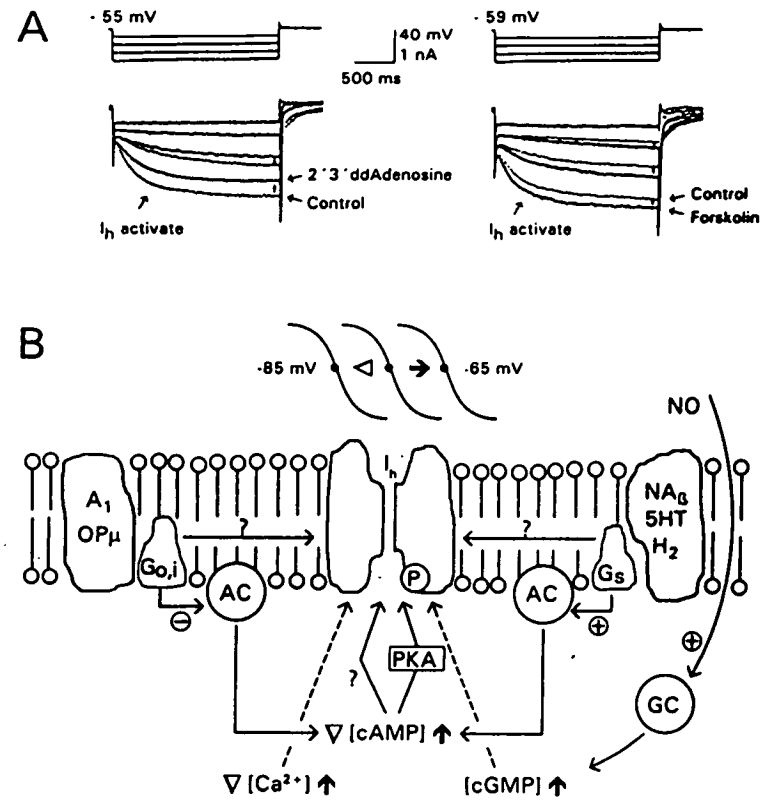

Figure 1 Regulation of I_h. (*A*) The I_h current in a thalamic neuron is decreased by the adenylyl cyclase (AC) inhibitor 2',3'-dideoxyadenosine and increased by the AC activator forskolin (modified from Reference 100). (*B*) Pathways of I_h regulation. Stimulation of receptors that are negatively (adenosine A_1, μ-opioid receptor) and positively (β-adrenergic, serotonergic, histamine H_2 receptor) coupled to AC activity induce a negative and positive shift in the I_h activation curve along the voltage axis, mediated via decreases and increases in the intracellular cAMP level, respectively. Whether cAMP directly modulates the channel or relies on PKA activity and phosphorylation (P) is undetermined. NO, through guanylyl cyclase (GC) activation and a rise in cGMP level, and presumably internal Ca^{2+}, regulate I_h activation via as yet unknown pathways (*dashed lines*). G_s and $G_{o,i}$ are stimulatory and inhibitory G proteins that may directly or indirectly influence the I_h channel. Stimulation, +; inhibition, −.

axis (Figure 1*B*), as has been shown for adenosine A_1 receptors in thalamic (100) and mesopontine neurons (105), and μ-opioid receptors in nodose ganglion cells (71). That this modulation occurs via adenylyl cyclase is indicated by two observations. First, opioids had no effect on I_h alone or

during the presence of cAMP analogues but reversed the effects of forskolin on I_h (71). Second, the negative shift in I_h activation upon A_1 receptor stimulation functionally antagonized the β-adrenergic response; it also occurred without prior activation of adenylyl cyclase activity and was imitated by the adenylyl cyclase inhibitor 2′,3′-dideoxyadenosine (Figure 1*A*), indicating mediation through a decrease in basal activity of adenylyl cyclase (100). A negative shift in I_h activation was also obtained with serotonin in cerebellar Purkinje cells (84), and there is evidence that hyperpolarization-activated currents are controlled through corticosteroid (77) or bradykinin action (80), although the mediating receptor subtypes and second messenger systems remain largely unknown. From these findings, it seems that the neuronal hyperpolarization-activated cation current is predominantly regulated through the basal activity of adenylyl cyclase and hence the intracellular concentration of cAMP. Stimulation of receptors that are negatively and positively coupled to adenylyl cyclase activity reciprocally shifts the I_h activation curve along the voltage axis, thereby controlling the availability of the I_h channels over a wide range of membrane potentials without changing the maximal conductance (Figure 1*B*). A similar relationship is known for cardiac sino-atrial node cells, where an opposite regulation of the cAMP level by muscarine or catecholamine regulates the voltage dependence of the equivalent current, I_f (17, 43–45). Studies of single I_f channels support this conclusion in that cAMP was found to shift the probability curve of first openings to shorter times without modification of the single-channel conductance (35, 39). Interestingly, as is the case in neurons, the I_f current in the heart is enhanced after stimulation of histamine H_2 receptors (109) and decreased during action of adenosine (108). Notable exceptions from this unifying principle include dopamine D_2 and $GABA_B$ receptors in ventral tegmental neurons, whose stimulation induces a reduction in maximal I_h current with no changes in kinetics or voltage dependence and with no apparent involvement of the adenylyl cyclase system (74).

The shift in the I_h activation curve suggests that receptor stimulation modulates I_h through an alteration in the voltage dependence of the underlying channels. Whether the change in voltage dependence results from activation of a cAMP-dependent protein kinase (PKA) cannot be deduced unambiguously from available data. The protein kinase inhibitor H-8 decreased the peak amplitude of I_h in dissociated bullfrog sympathetic neurons, which suggests channel regulation through protein kinase activity (125), but it did not influence β-adrenergic activation of I_h in thalamic neurons (100). A selective inhibitor of PKA (Walsh peptide) blocked the effect of β-adrenergic agonists and cAMP on the Ca^{2+}-dependent K^+ current I_{AHP}, but not on I_h, in hippocampal CA1 pyramidal cells, which suggested modulation of I_h in a kinase-independent manner (119a). Conflicting results were also obtained

from cardiac tissue. Two microelectrode voltage-clamp recordings in a multicellular Purkinje fiber preparation demonstrated that the cAMP-induced increase in I_f is reversed by protein kinase inhibition through H-7 and H-8, whereas application of H-8 in the absence of activators of the cAMP cascade does not influence the current, which suggests that phosphorylation of the channels takes place down-stream of cAMP action (21). However, recordings in inside-out macro-patches from enzymatically isolated sino-atrial node myocytes suggest a direct cAMP-dependent gating of the I_f channels independent of phosphorylation by a protein kinase (42): (*a*) The cAMP-induced shift of the activation curve occurred regardless of the presence of unspecific (H-7) or specific (pseudosubstrate peptide inhibitor) PKA inhibitors; (*b*) the application of PKA or active catalytic subunit did not activate the current; and (*c*) the action of addition or removal of cAMP in macro-patches was identical both qualitatively and quantitatively to the action of neurotransmitters on I_f in whole cells, thereby implying that cAMP by itself produces the range of changes induced by neurotransmitters. The exact reasons for these discrepancies remain unclear but may be related to the different experimental approaches, i.e. the use of inside-out macro-patches, which allows a strict control of substrate conditions, but which involves enzymes for dissociation of cells (cf 18), or dialysis by a patch pipette with heavily buffered intracellular Ca^{2+} level (cf 114), as opposed to the use of sharp microelectrodes in a multicellular preparation (21). However, the latter experiments that relied on the use of protein kinase inhibitors could not fully rule out the possibility of a direct blocking action of the inhibitors on the channels (cf 69) or of a reduction in I_h secondary to a lowered cAMP level due to a decrease in adenylyl cyclase activity upon generalized protein kinase inhibition (cf 106). An alternative explanation for the action of kinase inhibitors on I_h comes from the observation in rods (61) that the affinity of cGMP binding to light-activated channels is modulated by protein phosphatase activity. In conclusion, whether cAMP directly modulates the I_h channels or relies on protein kinase activity is still an open issue, although stronger evidence appears to favor a direct action of cAMP on the channels not involving protein phosphorylation.

There is also evidence from studies in excised membrane patches of sino-atrial node cells that the hyperpolarization-activated current is increased by the GTP-binding protein G_s and decreased by $G_{o,i}$, specifically by the α-subunits, which indicates that the channels may be directly regulated by the G proteins in addition to indirect cytoplasmic pathways via cAMP (140). The possible existence of a direct membrane delimited pathway between receptors and I_h channels in neurons is still unclear but appears unlikely for the following reasons: First, if adenosine A_1 receptors were directly coupled to I_h channels, the component of I_h modulation should have a time course similar to that for